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High expression of *N*-acetylglucosaminyltransferase V in favorable neuroblastomas: Involvement of its effect on apoptosis

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Abstract **Neuroblastoma (NBL), derived from the sympathetic precursor cells, is one of the most common pediatric solid tumors. The expression of *N*-acetylglucosaminyltransferase V and IX (GnT-V and GnT-IX) mRNA in 126 primary NBLs were quantitatively analyzed and higher expression levels of GnT-V were found to be associated with favorable stages (1, 2 and 4s). Conversely, the downregulation of GnT-V expression by small interfering RNA resulted in a decrease in the susceptibility to cell apoptosis induced by retinoic acid in NBL cells accompanied by morphological change. These results suggest that GnT-V is associated with prognosis by modulating the sensitivity of NBLs to apoptosis.**

Keywords: Neuroblastoma; GnT-V; GnT-IX; Retinoic acid; Apoptosis

1. Introduction

Aberrant glycosylation occurs in nearly all types of cancers, and has been implicated in the malignancy that is characteristic of the disease [1]. *N*-acetylglucosaminyltransferase V (GnT-V) is one of the most relevant glycosyltransferases to tumor invasion and metastasis, and catalyzes the formation of β1,6GlcNAc branching on *N*-glycans, which is closely associated with malignant transformations [2–6]. Recently, our group and Pierce's group independently reported on a new *N*-acetylglucosaminyltransferase IX (GnT-IX, also referred to as GnT-VB), a GnT-V homolog, that is specifically expressed in the brain [7,8]. GnT-IX transcripts are exclusively expressed in the brain and testis, while GnT-V is expressed ubiquitously in human and mouse tissues. Since both glycosyltransferases are expressed in the mouse brain in a region-specific manner (unpublished data), it is possible that they may have discrete biological functions in the brain. On the other hand, GnT-V and GnT-IX are both highly expressed in both the adult and fetal brain [7,9], as well as in several human neuroblastoma (NBL) cell lines (this study and unpublished data). This prompted us to examine the expression of GnT-V and GnT-IX in primary NBL tissues.

NBL is a tumor derived from primitive cells of the sympathetic nervous system and is the most common solid tumor in childhood [10]. Interestingly, most NBLs in infants regress spontaneously or mature into a benign ganglioneuroma. These tumors usually express high levels of TrkA, and as a result, have a tendency to either undergo apoptosis or differentiation, depending on whether nerve growth factor is present or absent in their microenvironment. On the other hand, in most patients over 1 year of age who have metastatic disease, the tumor grows aggressively and their prognosis is usually poor.

In this study, we carried out a quantitative analysis of the gene expression of these glycosyltransferases by real-time PCR, and the findings indicate that a higher expression of GnT-V is correlated with a favorable prognosis for NBL patients. Furthermore, to explore the underlying molecular mechanism, we devised a knockdown approach, in which small interfering RNA (siRNA)-directed against GnT-V mRNA was used to investigate the susceptibility to cell apoptosis induced by retinoic acid in NBL cells. The results clearly showed that the expression levels of GnT-V are associated with a favorable prognosis, possibly through sensitizing to apoptotic signals.

2. Materials and methods

2.1. RNA isolation from primary NBLs

Fresh, frozen tumor tissues were sent to the Division of Biochemistry, Chiba Cancer Center Research Institute, from various hospitals in Japan with informed consent from the patients' parents. All samples were obtained by surgery or biopsy and had been stored at 80 °C. The RNA samples obtained from 126 patients with NBL were subjected to semiquantitative and quantitative real-time reverse transcription-PCR (RT-PCR) analyses. All of the patients were diagnosed clinically as well as pathologically and were tested for DNA ploidy, MYCN amplification, and TrkA expression. The tumors were staged according to the criteria of the International Neuroblastoma Staging System [11].

2.2. Semiquantitative RT-PCR analysis of primary NBLs

The preparation of total RNA from NBL tissues and the synthesis of the first-strand cDNA were performed as described previously [12]. The cDNA was diluted to a 1:20 solution and then amplified in a final

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Abbreviations: NBL, neuroblastoma; GnT-V, *N*-acetylglucosaminyltransferase V; GnT-IX, *N*-acetylglucosaminyltransferase IX; RT, reverse transcription; PARP, poly(ADP-ribose) polymerase

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volume of 10 μl of reaction mixture containing 200 μM of dNTPs, 1× PCR buffer, 0.5 μM of each primer and 0.2 U of rTaq DNA polymerase (Takara Bio, Ohtsu, Japan). The following primer sets were used: GnT-V, 5′-GACCTGCAGTTCCTTCTTCG-3′ and 5′-CCATGGCAGAAGTCCTGTTT-3′; GnT-IX, 5′-CATGGCACCGTGTACTAC-3′ and 5′-TCTGGAGCTCTGCAGAAG-3′. PCR templates were standardized by their GAPDH expression before performing the RT-PCR experiments.

2.3. Quantitative real-time PCR analysis of primary NBLs

2 μl of cDNA prepared as above, either a 100-fold dilution for GnT-V or a 20-fold dilution for GnT-IX, was amplified in a volume of 20 μl with Assay-on-Demand Gene Expression Products (Applied Biosystems) consisting of primers and a TaqMan probe (Assay ID: GnT-V, Hs00159136_m1; GnT-IX, Hs01586304_g1). The thermal cycling conditions and the normalization of the data using GAPDH expression were performed as described previously [12]. All experiments were carried out in triplicate for each data point.

2.4. Assay of GlcNAc transferase activity

The activities of GnT-V and GnT-III in whole cell lysates or microsomal fractions were determined using a pyridylaminated biantennary sugar chain as an acceptor substrate, as described previously [7,13].

2.5. Construction of siRNA vector and retroviral infection

Small interfering oligonucleotides specific for GnT-V were designed on the Takara Bio website (http://www.takara-bio.co.jp/) and the oligonucleotide sequences used in the construction of the siRNA vector were as follows: 5′-GATCCGTTCATTGGCGGAAATTCGTTTCAAGAGAACGAATTTCCGCCAATGAACTTTTTTAT-3′ and 5′-CGATAAAAAAGTTCATTGGCGGAAATTCGTTCTCTTGAAACGAATTTCCGCCAATGAACG-3′. The oligonucleotides were annealed and then ligated into *Bam*HI/*Cla*I sites of the pSINsi-hU6 vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using a Retrovirus Packaging Kit Ampho (Takara Bio) according to the manufacturer's protocol. CHP134 cells, a human NBL cell line, were infected with the viral supernatant, and the cells were then selected with 0.5 mg/ml G418 for 2–3 weeks. Stable GnT-V-knockdown clones were selected and confirmed by GnT-V activity and gene expression. Quantitative real-time PCR analyses of GnT-V mRNA expression in these clones were performed with a Smart Cycler II System and the SYBR premix Taq (Takara Bio). RT was carried out at 42 °C for 10 min, followed by 95 °C for 2 min using random primers, followed by PCR for 50 cycles at 95 °C for 5 s and 60 °C for 20 s with the following primers: 5′-AAGCAGGTGTGCCAGGAGAG-3′ and 5′-GTCAAAGGAGGGCACCAGGA-3′. Normalization of the data was performed using the GAPDH mRNA levels.

2.6. Analysis of retinoic acid-induced apoptosis

Parent, mock, and GnT-V-knockdown CHP134 cells were plated on 10 cm culture dishes at 5×10^5 cells in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After incubation for 24 h, the conditioned media were changed with fresh medium containing various concentrations of all-*trans* retinoic acid (Sigma). The cells were washed twice with PBS and harvested at the indicated times. Retinoic acid-induced apoptosis was estimated by detecting the cleavage of poly ADP-ribose polymerase (PARP) in whole cell lysates by Western blot analysis using a human specific anti-cleaved PARP (Asp214) antibody (Cell Signaling Technology). As a loading control, anti-ERK1/2 (p44/42 MAP Kinase Antibody, Cell Signaling Technology) was used.

2.7. Viability assay of retinoic acid-treated cells

The parent, mock, and GnT-V-knockdown CHP134 cells were seeded on a 96-well plate at 3×10^3 cells/well for 24 h prior to the retinoic acid treatment. The cells were then incubated with or without retinoic acid at the indicated concentrations for 3 days. Cell viability was assayed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacture's instructions. All experiments were carried out in triplicate for each data point.

3. Results

3.1. Association between higher expression levels of GnT-V mRNA and favorable prognosis in primary NBLs

To assess the association between GnT-V or GnT-IX mRNA expression and the prognosis of the NBLs, we first performed semiquantitative RT-PCR analyses using 16 favorable and 16 unfavorable NBLs. As shown in Fig. 1, GnT-V was preferentially expressed in most of the favorable NBLs, while no obvious difference in GnT-IX expression was found between favorable and unfavorable NBLs. Table 1 shows quantitative data for GnT-V and GnT-IX mRNA in 126 primary NBLs with tumor stages (1, 2, 4s versus 3, 4). GnT-V expression was significantly increased in NBLs at favorable stages ($P = 0.021$), and was correlated well with higher expression of TrkA ($P = 0.010$). On the other hand, GnT-IX expression was marginally associated with the stages.

3.2. GnT-V activities in various human NBL cell lines

To determine whether the expression level of GnT-V is also increased in NBL cells, the activities of GnT-V in various human NBL cell lines were examined. As shown in Fig. 2A, each cell line expressed GnT-V activity at distinct levels. The CHP134 cells showed the highest GnT-V activity among the 10 NBL cell lines used in this study. It is known that the cell line is highly sensitive to the induction of apoptosis by all-*trans* retinoic acid [14,15]. In fact, it is thought that favorable NBLs usually express higher levels of TrkA, and tend to regress spontaneously due to apoptosis. As shown in Fig. 2B, in CHP134 cells that had been treated with retinoic acid at a concentration of 1 μM or 5 μM, PARP cleavage, a marker for apoptosis, occurred, which is one of the main cleavage targets of caspase-3

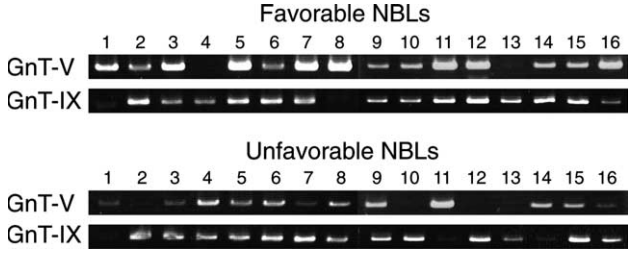

Fig. 1. Semiquantitative RT-PCR analysis of favorable and unfavorable subsets of NBL. Sixteen favorable cases were in stage 1 with no MYCN amplification and a high TrkA expression, while 16 unfavorable cases were in stage 3 or 4 with MYCN amplification and a low TrkA expression.

Table 1
Association of tumor stages and *TrkA* expression in NBL patients with GnT-V or GnT-IX mRNA expression levels

	n	GnT-V[a]	*P*	GnT-IX[a]	*P*
Tumor stage					
1, 2, 4s	57	2.23 ± 0.29	0.021	1.78 ± 0.25	0.21
3, 4	69	1.48 ± 0.16		2.23 ± 0.25	
TrkA expression					
High	59	2.11 ± 0.27	0.010	1.86 ± 0.17	0.75
Low	48	1.33 ± 0.13		1.98 ± 0.34	

[a]Means ± S.E.M.

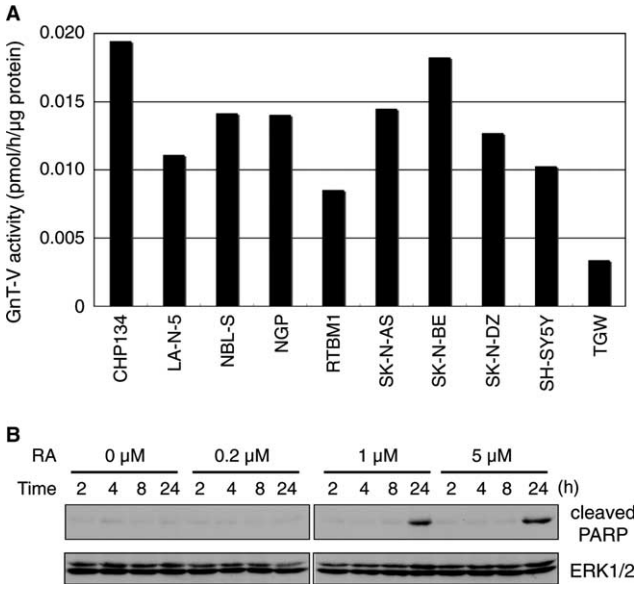

Fig. 2. GnT-V activities in various NBL cell lines and retinoic acid-induced apoptosis of CHP134 cells. (A) GnT-V activity of each of the NBL cells was measured using a whole cell lysate as an enzyme source. (B) Western blot of whole cell lysate of CHP134 cells. Cell apoptosis was observed by staining of cleaved PARP after treatment of retinoic acid (RA) at indicated concentrations and times. The expression levels of ERK1/2 were confirmed as a loading control.

in vivo [16,17]. Thus, we chose this cell line for further analysis of the effects of GnT-V on apoptosis.

3.3. Knockdown of GnT-V expression in CHP134 cells

We prepared a retroviral siRNA vector containing a small hairpin construct capable of generating a duplex RNAi oligonucleotide corresponding to human GnT-V. After retroviral infection, CHP134 cells were selected based on their resistance to G418, and clones with decreased GnT-V activities were chosen. The GnT-V activities were effectively downregulated by 80%, compared with those in parent or mock cells (Fig. 3A), while GnT-III activity, as a control, showed no significant changes between those cells. A quantitative real-time PCR analysis also indicated the downregulation of RNAi-directed GnT-V mRNA expression in these cells (Fig. 3B). It is noteworthy that the cells in GnT-V-knockdown clones showed more spreading on the culture dishes, rather than the spindle shapes of the parent and mock cells (Fig. 4), suggesting that GnT-V may affect cellular cytoskeletal formation. In fact, Guo et al. reported that the overexpression of GnT-V in human HT1080 cells resulted in a decrease in cell adhesion on fibronectin [18].

3.4. Decreased susceptibility to retinoic acid-induced apoptosis in GnT-V-knockdown cells

To evaluate the effects of GnT-V expression on susceptibility to apoptosis induction in CHP134 cells, we examined cell viabilities in the presence of retinoic acid. After treatment with different concentrations of retinoic acid, we found that GnT-V-knockdown cells (KD1 and KD2 in Fig. 5A) had a tendency to be resistant to stimulation by retinoic acid. We further assessed the apoptosis level in retinoic acid-treated cells by PARP cleavage. The GnT-V-knockdown cells showed dramatically reduced levels of PARP cleavage (Fig. 5B). Collectively, these results suggest that GnT-V may sensitize cells to apoptotic signals, which partly contribute to the favorable prognosis of NBL.

4. Discussion

Previous studies demonstrated that an increased amount of β1,6-branched oligosaccharides, formed by the action of GnT-V, are correlated with metastatic potential [2], and this has been shown to be a marker of tumor progression in human breast and colon neoplasia [19], and a prognostic marker in human colorectal carcinoma [20,21]. However, it is not always the case, as evidenced by the fact that Dosaka-Akita et al. reported that the lower expression of GnT-V is associated with a shorter survival and a poor prognosis in non-small cell lung cancers [22]. The present study also suggested that a higher expression of GnT-V is related to a favorable prognosis in NBLs.

GnT-V and GnT-IX, two closely related glycosyltransferases, are expressed in both the adult and fetal brain [7,9]. GnT-V expression is upregulated in E9.5 embryos, and is then

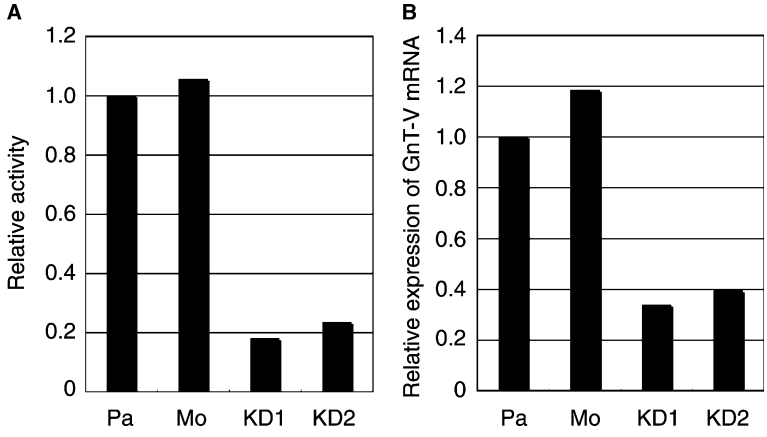

Fig. 3. Enzyme activities and mRNA expression levels in siRNA-mediated GnT-V-knockdown cells. (A) GnT-V activities of GnT-V-knockdown CHP134 cells. The microsomal fraction was used as an enzyme source in the assay. (B) mRNA expression of GnT-V in knockdown cells. Quantitative analysis was performed by real-time PCR. Pa, parent cells; Mo, mock cells; KD1 and KD2, GnT-V-knockdown cells.

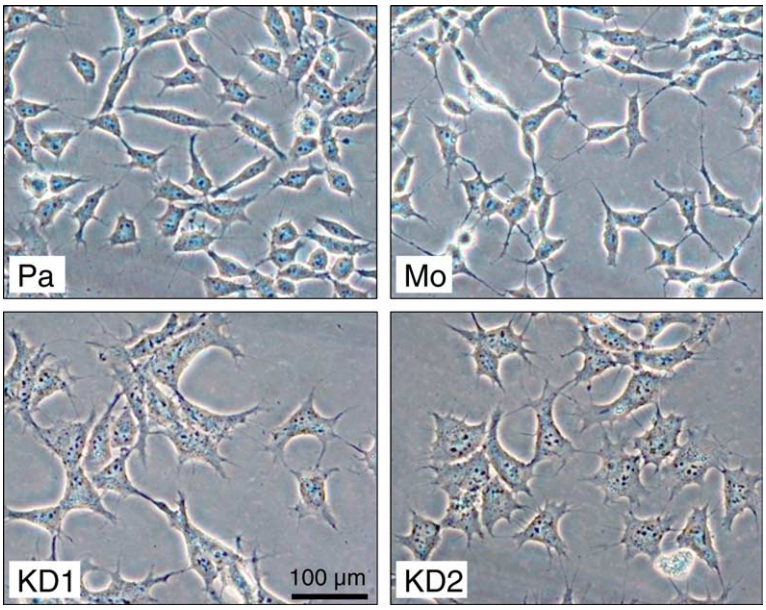

Fig. 4. Morphological changes in GnT-V-knockdown cells. Parent (Pa), mock (Mo), and GnT-V-knockdown CHP134 cells (KD1, KD2) were plated on culture dishes and incubated for 24 h in culture media. Cell shapes were observed by phase contrast microscopy.

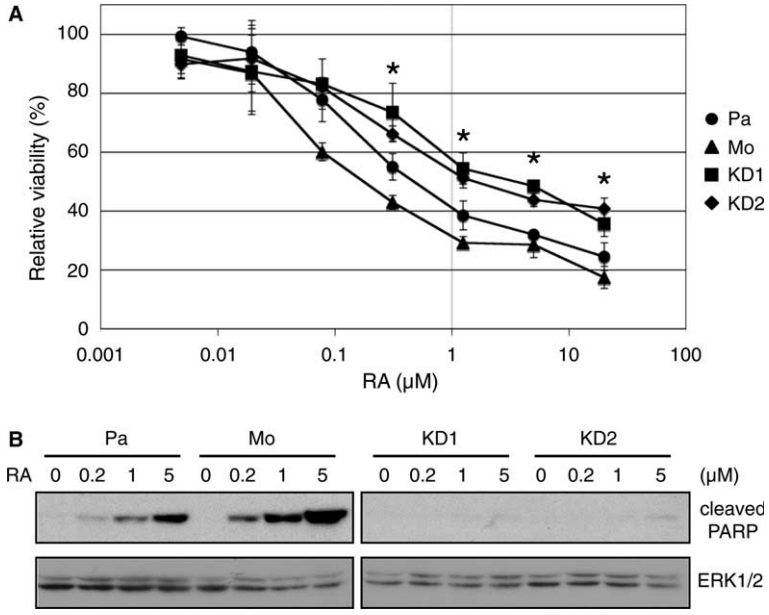

Fig. 5. Cell viabilities and PARP cleavage in retinoic acid-treated GnT-V-knockdown cells. Cell viabilities of parent (Pa), mock (Mo), and GnT-V-knockdown cells (KD1, KD2) were performed as described in Section 2 (A). Cells were treated with retinoic acid at the indicated concentrations for 3 days. $^{*}P < 0.05$. (B) Western blot of cleaved PARP in a cell lysate using an anti-cleaved PARP antibody. Cells were harvested for analysis 24 h after retinoic acid-treatment.

restricted to regions comprised of several specialized epithelial cell layers and the neuroepithelium of the developing central nervous system [9]. On the other hand, GnT-IX is dominantly expressed in the human and mouse brain [7,23]. Thus, we attempted to examine the expressions of GnT-V and GnT-IX in primary NBL tissues.

The frequent gain of the chromosome 17q has been reported to be associated with a poor prognosis [24], and the preferential gain of the region from 17q22-qter indicated a dosage effect that provides a selective advantage to be aggressive NBLs [25]. The gene responsible for the selective advantage is unknown, but a candidate gene that is a member of the inhibitor of apoptosis proteins, survivin, which is mapped to 17q25, has been reported [14]. Although the GnT-IX gene is also mapped to 17q25 [7], an unequivocal correlation with prognosis was not observed in this study. Interestingly, a significant association between expression levels of GnT-V mRNA and the prognosis of 126 NBL patients was observed by real-time PCR analysis. Several human NBL cell lines also consistently express GnT-V. To understand the molecular mechanism associated with the

higher expression levels of GnT-V in the favorable prognosis of NBLs, we selected CHP134 cells as a cell model. Since the cell line is highly sensitive to retinoic acid-induced apoptosis [14,15], we compared the effects of retinoic acid on apoptosis between parent cells and GnT-V-knockdown cells.

In fact, GnT-V-knockdown cells showed a tendency to escape from retinoic acid-induced apoptosis, as confirmed by a cell viability assay and the extent of cleaved PARP, supporting the notion that a higher expression of GnT-V is correlated with a favorable prognosis of NBLs. It is noteworthy that a prominent morphological alteration with increased spreading was observed in the GnT-V-knockdown cells. The altered characteristic of GnT-V-knockdown CHP134 cells observed in this study is consistent with those of previous studies [3,18,26,27]. The overexpression of GnT-V enhances the metastatic potential in several cell types with reduced cell-matrix adhesion and increased motility [3,18,26]. Furthermore, GnT-V expression in human glioma cell line U-373 MG sensitizes these cells to drag-induced apoptosis [28]. Conversely, GnT-V null mouse embryonic fibroblasts exhibited an enhanced adhesion and spreading with associated reduced cell migration [27]. In addition, no significant effect of GnT-V overexpression was observed on apoptotic behavior in fibrosarcoma HT1080 cells, a fibroblast cell line, but a similar phenotypic change with regard to adhesion and migration has been reported [18]. In general, the adhesion of epithelial cells to extracellular matrices is weaker than that of fibroblast cells, and such adhesion is thought to be synergized with the signals of growth factor receptors for modulating cell proliferation and apoptosis. Therefore, we speculate that the GnT-V-induced decrease in cell adhesion could be a plausible factor responsible for the favorable prognosis in NBLs.

In conclusion, a correlation between higher expression levels of GnT-V with a favorable prognosis of NBL patients was found, and GnT-V may cause these tumors to regress by increasing their susceptibility to apoptosis.

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